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NOTES

PtrA Is a Periplasmic Protein Involved in Cu Tolerance in *Pseudomonas aeruginosa*▽†

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In this work, we demonstrate that PtrA (U. H. Ha et al., Mol. Microbiol. 54:307–320, 2004) is a periplasmic protein, specifically synthesized in the presence of copper, that is involved in the tolerance of *Pseudomonas aeruginosa* to copper. Our biochemical and genetic analyses challenge its role in transcriptional inhibition of the type III secretion system.

The PtrA (*Pseudomonas* type III repressor A) protein (5) was previously identified as a specific inhibitor of ExsA, the key activator of the type III secretion system (T3SS) in *Pseudomonas aeruginosa* (3, 11, 13). Identified by *in vivo* expression technology applied to a mouse burn infection model, overexpression of *ptrA* was further shown to specifically inhibit the T3SS genes in a gene expression microarray experiment (5). A direct interaction between PtrA and ExsA was further proposed using two-hybrid, enzyme-linked immunosorbent assay (ELISA), and pulldown experiments (5). The *ptrA* gene (*PA2808*) is divergently transcribed from the *copR-copS* operon, which encodes a two-component regulatory system involved in copper resistance and is required for *ptrA* transcription in response to copper (5). PtrA was thus proposed to be an inhibitor of ExsA that represses T3SS synthesis in response to copper stress.

The transcription factor ExsA is an activator of the AraC/XylS family; it positively autoregulates its own transcription and is inhibited by the antiactivator ExsD, which belongs to a regulatory pathway coupling T3 secretory activity and gene transcription (2, 7). The molecular mechanism underlying the inhibition of ExsA by ExsD has been extensively studied by several groups, including ours. By sequestering the transcriptional activator in a 1:1 complex, ExsD inhibits the self-association properties and the DNA binding activity of ExsA and, consequently, the transcription of T3S operons (1, 10). Eager to elucidate the mechanism employed by PtrA to inhibit ExsA transcriptional activity, we undertook experiments to characterize the ExsA/PtrA complex in terms of stoichiometry and activity, as was done previously for the ExsA/ExsD complex (10). However, in contradiction with previously reported data (5), no evidence of an ExsA/PtrA interaction could be obtained using a pulldown assay, an ELISA, or an adenylate cyclase-based bacterial two-hybrid system (see Fig. S1 in the supplemental material). Furthermore, all attempts to detect a complex by nickel affinity chromatography with proteins produced in *Escherichia coli* failed (data not shown).

Therefore, we undertook physiological studies to gain insight into the role of PtrA in *P. aeruginosa*. A fusion between the promoter of *ptrA* and the *lacZ* reporter gene was inserted into the chromosome of *P. aeruginosa* (see the supplemental material). *ptrA* promoter activity was strongly induced by 2 mM $CuSO_4$, as previously reported (5), whereas no activity was measured either in buffered LB medium (LBM) or under con-

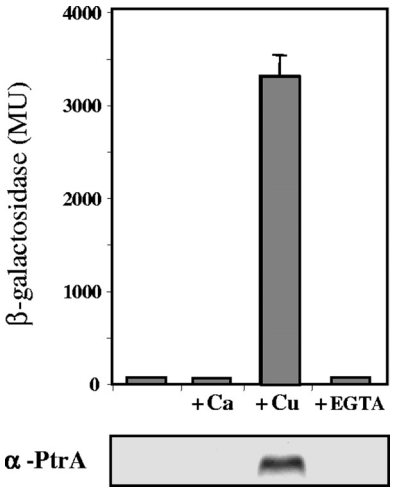

FIG. 1. PtrA is synthesized in the presence of copper. The histogram represents the β-galactosidase activity (expressed in Miller units [MU]) from the *P. aeruginosa* P*ptrA-lacZ* strain grown in LBM containing 5 mM $CaCl_2$ (+Ca), 2 mM $CuSO_4$ (+Cu), or 5 mM EGTA-20 mM $MgCl_2$ (+EGTA). Error bars denote standard deviations. The lower panel represents the anti-PtrA immunoblot analyses of the total soluble fractions of the corresponding cells.

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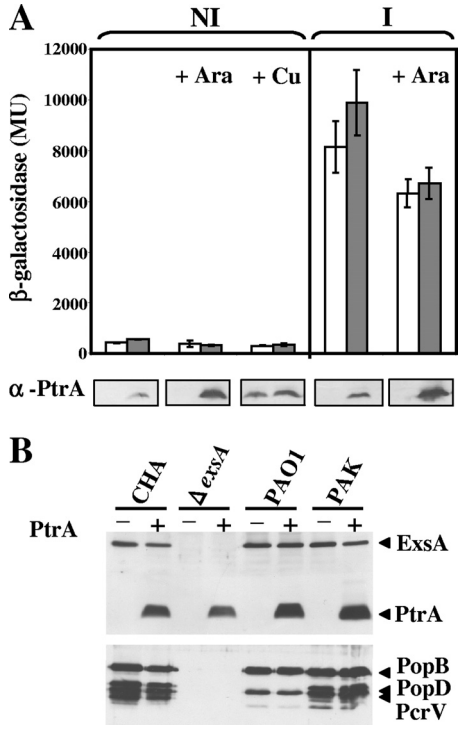

FIG. 2. PtrA does not affect ExsA and T3SS activity. (A) The histogram represents the β-galactosidase activity from the *P. aeruginosa* P_G-*lacZ* strain harboring pJN105 (white bars) or pJN-PtrA (gray bars). Cells were grown in LBM either under T3SS-noninducing (NI) or -inducing (I) conditions, and 0.5% arabinose (+Ara) or 2 mM $CuSO_4$ (+ Cu) was added where indicated. Of note, the "I + Cu" condition was not tested, as EGTA chelates Cu^{2+}. Error bars denote standard deviations. The lower panels represent the anti-PtrA immunoblot analyses of the soluble fractions of the corresponding cells. (B) Immunoblot analyses of the CHA, Δ*exsA*, PAO1, and PAK strains containing either the pJN105 (−) or pJN-PtrA (+) plasmid. The cells were grown under T3SS-inducing conditions in the presence of 2% arabinose. The upper panel corresponds to the soluble protein fractions resolved in a 15% acrylamide denaturing gel and blotted with anti-ExsA and anti-PtrA. The lower panel represents the T3S secreted proteins, separated onto a 13.5% acrylamide denaturing gel and revealed with anti-PopB, anti-PopD, and anti-PcrV.

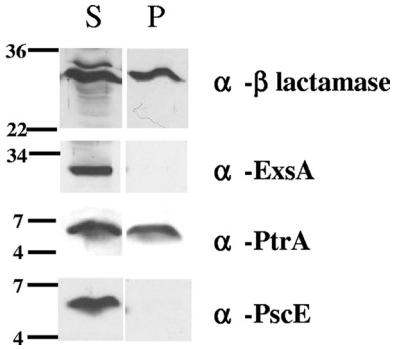

FIG. 3. PtrA is a periplasmic protein. Western blot analysis of β-lactamase, ExsA, PtrA, and PscE levels in the total soluble (S) and periplasmic (P) fractions. The proteins were separated in a 15% acrylamide gel prior to electrotransfer. The protein markers on the left are indicated in kDa.

ditions used to activate (Ca depletion) or repress (Ca addition) T3SS gene expression. These results were confirmed at the protein level by using anti-PtrA antibodies raised against the recombinant His_{10}-tagged protein (Fig. 1). As the endogenous protein was not synthesized at detectable levels under conditions commonly used to trigger T3SS expression, we placed the *ptrA* gene under the control of the P_{BAD} arabinose-inducible promoter (pJN-PtrA) and induced PtrA synthesis in the wild-type strain containing a chromosomal fusion of the ExsA target promoter P_G (promoter of the *pcrGVH-popBD* operon) with *lacZ* (see the supplemental material). β-Galactosidase activities were then measured under different conditions inducing PtrA and/or T3SS, and the presence of PtrA was monitored by Western blotting. As shown in Fig. 2A, the chromosomally encoded (Cu-induced) and/or plasmid-encoded (arabinose-induced) PtrA had no effect on basal ExsA activity under T3SS-repressing conditions (noninducing [NI] condition) or on ExsA activity stimulated by Ca depletion (inducing [I] condition). As the PtrA-reported effect on T3SS genes was previously observed in *P. aeruginosa* strain PAK (5), we introduced the pJN-PtrA plasmid in the PAK, PAO1, and CHA strains of *P. aeruginosa*, as well as in a CHA Δ*exsA* mutant. The activity of ExsA was assessed by the secretion of three T3S proteins, PopB, PopD, and PcrV (4), whose synthesis is dependent on ExsA (12). Figure 2B clearly shows that overproduced PtrA did not affect the T3S translocator secretion in all tested strains and that the absence of PtrA's effect on T3SS is not strain dependent. Finally, we used the T3SS-dependent cytotoxicity toward macrophages (3) as an independent measure of the T3SS activity of different strains expressing or not expressing PtrA. Here again, no effect of PtrA could be detected (data not shown). Taking into account our biochemical analysis as well as *in vivo* approaches shown in Fig. 1 and 2, we conclude that PtrA does not play any role in the regulation of ExsA activity or in T3SS synthesis. The discrepancy between our result and the study by Ha et al. (5) might come from the plasmid used to express *ptrA*: in the earlier report (5), the pUCP19-derived plasmid (pHW0141) contained a 1.2-kb DNA fragment, much longer than the 192-bp-long *ptrA* gene. Unlike in our experiments, synthesis of PtrA from this plasmid was not demonstrated, and other elements (such as a putative truncated CopR) might be encoded by the DNA fragment and responsible for the effect observed on the T3SS in *P. aeruginosa.*

PtrA is a small (63-amino-acid [aa]), basic (pI 9) protein predicted to fold in 2 α-helices (PSIPRED), the C-terminal one (aa 28 to 61) being a putative coiled coil (COILS). A type I signal peptide of 23 aa is predicted at its N terminus (6), strongly suggesting that PtrA could localize to the periplasm. To establish the PtrA localization in *P. aeruginosa*, cell fractionation experiments followed by immunoblotting were performed, using β-lactamase as a periplasmic marker. As shown in Fig. 3, both β-lactamase and PtrA were found in the periplasmic fraction, whereas ExsA and a 7.3-kDa T3SS chaperon, PscE (8), both used as controls, were present only in the cytosolic fraction. Therefore, these data show that PtrA is a periplasmic protein.

The *ptrA* gene is located upstream from *PA2807* (see Fig. S2A in the supplemental material), which is strongly upregulated in response to Cu, as observed for *ptrA* (9). *PA2807* codes

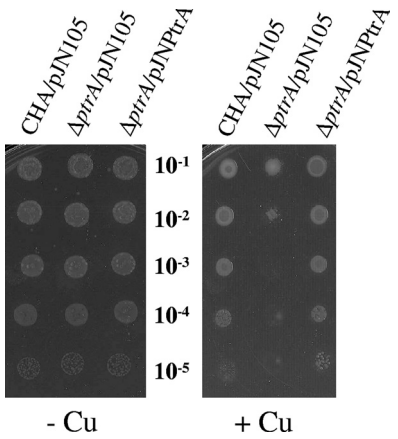

FIG. 4. PtrA is involved in Cu resistance. The wild-type and Δ*ptrA* strains containing pJN105, as well as a Δ*ptrA* strain harboring pJN-PtrA, were grown in the presence of 0.5% arabinose. Then 10 μl of a 10-fold serial dilution of the three cultures was spotted on M9 medium supplemented with 0.2% glucose and containing 1 mM $CuSO_4$ where indicated. The plates were incubated at 37°C for 16 h (no Cu) or 40 h (1 mM $CuSO_4$).

for a putative member of the plastocyanin/azurin copper-binding family of proteins that possesses a predicted export sequence signal of type I. A *PA2807* mutant presents a slight increase in copper sensitivity compared to the wild-type strain in a Cu disk sensitivity assay, pointing to a probable role in copper tolerance of the corresponding protein (9). *ptrA* is located next to the genes encoding the CopR/CopS two-component regulatory system, which controls *ptrA* expression in response to copper (6) and which plays an important role in Cu tolerance (9). The PtrA sequence is highly conserved in all strains of *P. aeruginosa* with no obvious orthologs in other pseudomonads; however, small proteins harboring homology with PtrA (around 25% identity) that are predicted to localize to the periplasm (see Fig. S2B) are encoded in the genomes of numerous *Pseudomonas putida* strains and in *Pseudomonas mendocina* strain ymp. These genes are found to be linked to genes encoding two-component regulatory systems involved in heavy metal responses or to genes assigned as encoding predicted copper resistance proteins of the CopA family (http://www.pseudomonas.com/) (see Fig. S2A). Therefore, a role of PtrA in Cu resistance/tolerance of *P. aeruginosa* was investigated by testing the sensitivity to copper of wild-type and Δ*ptrA* strains, as well as of the complemented mutant (Δ*ptrA*/pJN-PtrA), on minimal medium (Fig. 4). The sensitivity of the mutant, compared to that of the wild-type strain, was clearly increased, a phenotype that was corrected by the presence of the plasmid-encoded PtrA protein (Fig. 4). Of note, the three strains exhibited the same sensitivity toward other divalent cations, such as Ni^{2+} or Mn^{2+} (data not shown).

All together, our results indicate that the PtrA protein is specifically induced by copper, is found in the periplasm, and plays a role in copper tolerance in *P. aeruginosa*. As we questioned its role in T3SS regulation, its precise physiological role needs to be further characterized.

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